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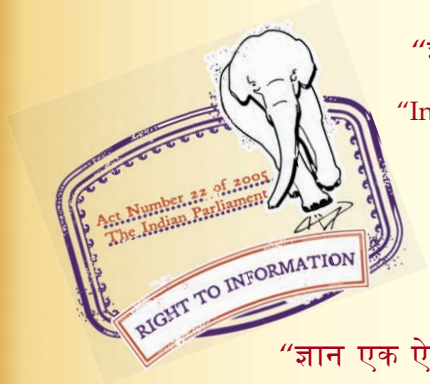
IS 11202 (2005): Dried milk - Determination of Lactic acid and lactates content - Enzymatic method [FAD 19: Dairy Products and Equipment]



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"ज्ञान एक ऐसा खजाना है जो कभी चुराया नहीं जा सकता है"
Bhartṛhari—Nītiśatakam
"Knowledge is such a treasure which cannot be stolen"

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IS 11202 : 2012
ISO 8069 : 2005

भारतीय मानक

शुष्क दुग्ध — लेक्टिक अम्ल और लेक्टेट्स का अंश ज्ञात करना

(दूसरा पुनरीक्षण)

Indian Standard

DRIED MILK — DETERMINATION OF CONTENT OF LACTIC ACID AND LACTATES

(Second Revision)

ICS 67.100.10

BUREAU OF INDIAN STANDARDS
MANAK BHAVAN, 9 BAHADUR SHAH ZAFAR MARG
NEW DELHI 110002

December 2012 **Price Group 7**

Dairy Products and Equipment Sectional Committee, FAD 19

NATIONAL FOREWORD

This Indian Standard (Second Revision) which is identical with ISO 8069 : 2005 'Dried milk — Determination of content of lactic acid and lactates' issued by the International Organization for Standardization (ISO) was adopted by the Bureau of Indian Standards on the recommendation of the Dairy Products and Equipment Sectional Committee and approval of the Food and Agriculture Division Council.

This standard was first published in 1984 which was identical with ISO 3495 : 1975 under dual numbering system. Subsequently ISO 3495 was withdrawn and replaced by ISO 8069 : 1986. The first revision was brought out in 2005 to align it with ISO 8069 : 1986. The second revision of this standard has been undertaken to align with the latest version of ISO 8069 : 2005, which has been technically revised.

The text of ISO Standard has been approved as suitable for publication as an Indian Standard without deviations. Certain conventions are, however, not identical to those used in Indian Standards. Attention is particularly drawn to the following:

a) Wherever the words 'International Standard' appear referring to this standard, they should be read as 'Indian Standard'.
b) Comma (,) has been used as a decimal marker while in Indian Standards, the current practice is to use a point (.) as the decimal marker.

In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS 2 : 1960 'Rules for rounding off numerical values (*revised*)'.

Indian Standard

DRIED MILK — DETERMINATION OF CONTENT OF LACTIC ACID AND LACTATES

(Second Revision)

1 Scope

This International Standard specifies an enzymatic method for the determination of the lactic acid and lactates content of all types of dried milk.

2 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

2.1
lactic acid and lactates content
mass of substances determined by the procedure specified in this International Standard

NOTE It is expressed as milligrams of lactic acid per 100 g of non-fat solids.

3 Principle

A test portion of dried milk is dissolved in warm water. The fat and proteins are precipitated then filtered. The filtrate is treated with the following enzymes and biochemical substances, added simultaneously, but acting in sequence:

a) L-lactate dehydrogenase (L-LDH) and D-lactate dehydrogenase (D-LDH), in the presence of nicotinamide adenine dinucleotide (NAD), to oxidize lactate to pyruvate and to convert NAD to its reduced form NADH;

b) glutamate pyruvate transaminase (GPT), in the presence of L-glutamate, to transform pyruvate into L-alanine and to convert L-glutamate to α-ketoglutarate.

The amount of NADH produced is determined by spectrophotometric measurement at a wavelength of 340 nm, and is proportional to the lactic acid and lactates content.

4 Reagents

Use only reagents recognized analytical grade. The water used in the preparation of the enzyme solutions shall be of at least doubly glass-distilled purity and the water used for other purposes shall be glass-distilled or of at least equivalent purity.

4.1 Potassium hexacyanoferrate(II) solution, $c(K_4[Fe(CN)_6]\cdot 3H_2O)$ = 35,9 g/l.

Dissolve 35,9 g of potassium hexacyanoferrate(II) trihydrate in water. Dilute with water to 1 000 ml and mix.

4.2 Zinc sulfate solution, $c(ZnSO_4\cdot 7H_2O)$ = 71,8 g/l.

Dissolve 71,8 g of zinc sulfate heptahydrate in water. Dilute with water to 1 000 ml and mix.

4.3 Sodium hydroxide solutions

4.3.1 Sodium hydroxide solution I, c(NaOH) = 10 mol/l.

Dissolve 400 g of sodium hydroxide in water. Dilute with water to 1 000 ml and mix.

4.3.2 Sodium hydroxide solution II, c(NaOH) = 0,1 mol/l.

Dissolve 4,0 g of sodium hydroxide in water. Dilute with water to 1 000 ml and mix.

4.4 Glycerol solution ($C_3H_8O_3$), with a volume fraction of 50 % glycerol.

4.5 Ammonium sulfate solution, $c[(NH_4)_2SO_4]$ = 3,2 mol/l.

Dissolve 422,84 g of ammonium sulfate in water. Dilute with water to 1 000 ml and mix.

4.6 Buffer solution, pH 10.

Dissolve 7,92 g of glycylglycine ($C_4H_8N_2O_3$) and 1,47 g of L-glutamic acid ($C_5H_9NO_4$) in about 80 ml of water. Adjust the pH to 10,0 $\pm$ 0,1 at 20 °C with sodium hydroxide solution I (4.3.1). Dilute with water to 100 ml and mix.

This solution may be kept for 3 months if stored in a refrigerator at between 0 °C and +5 °C.

4.7 Nicotinamide adenine dinucleotide solution (NAD).

Dissolve 350 mg of nicotinamide adenine dinucleotide ($C_{21}H_{27}N_7O_{14}P_2$) in 10 ml of water.

This solution may be kept for 4 weeks if stored in a refrigerator at between 0 °C and +5 °C. When the solution is being used, keep the vessel immersed in crushed ice.

4.8 L-Lactate dehydrogenase (L-LDH), from hog muscle suspension.

Dissolve 10 mg of L-lactate dehydrogenase suspension in 1 ml of glycerol solution (4.4). The pH of the obtained suspension should be about 7. The specific activity of the L-lactate dehydrogenase (L-LDH, EC 1.1.1.27) suspension shall be at least 5 500 units/ml at 25 °C. If not, prepare another L-LDH suspension.

The L-LDH suspension may be kept for 12 months if stored in a refrigerator at between 0 °C and +5 °C. When the suspension is being used, keep the vessel immersed in crushed ice.

4.9 D-Lactate dehydrogenase (D-LDH), from *Lactobacillus leichmannii* suspension.

Dissolve 5 mg of D-LDH suspension in 1 ml of ammonium sulfate solution (4.5). The pH of the obtained suspension should be about 6. The specific activity of the D-lactate dehydrogenase (D-LDH, EC 1.1.1.28) suspension shall be at least 1 500 units/ml at 25 °C. If not, prepare another D-LDH suspension.

The D-LDH suspension may be kept for 12 months if stored in a refrigerator at between 0 °C and +5 °C. When the suspension is being used, keep the vessel immersed in crushed ice.

4.10 Glutamate pyruvate transaminase (GPT), from pig heart suspension.

Dissolve 20 mg of GPT suspension in 1,0 ml of ammonium sulfate solution (4.5). The pH of the obtained suspension should be about 7. The specific activity of the glutamate pyruvate transaminase (GPT, EC 2.6.1.2) suspension shall be at least 1 600 units/ml at 25 °C. If not, prepare another GPT suspension.

Add 1,0 ml of ammonium sulfate solution (4.5) to the 1 ml suspension with 20 mg of GPT and mix. Centrifuge this 2,0 ml suspension containing 10 mg of GPT/ml at a radial acceleration of 4 000 g for 10 min. Transfer 1,0 ml of the clear supernatant liquid and discard the remaining solution and pellet.

The suspension may be kept for 12 months if stored in a refrigerator at between 0 °C and +5 °C. When the suspension is being used, keep the vessel immersed in crushed ice.

4.11 Lithium L-lactate solution

Dissolve 50 mg of lithium L-lactate ($C_3H_5O_3Li$) in water. Dilute with water to 500 ml and mix.

4.12 Lithium D-lactate solution

Dissolve 50 mg of lithium D-lactate ($C_3H_5O_3Li$) in water. Dilute with water to 500 ml and mix.

5 Apparatus

Usual laboratory equipment and, in particular, the following.

5.1 Analytical balance, capable of weighing to the nearest 1 mg, with a readability of 0,1 mg.

5.2 Glass beaker, of capacity 50 ml.

5.3 Graduated cylinder, of capacity 50 ml.

5.4 One-mark volumetric flasks, of capacity 100 ml.

5.5 Pipettes, capable of delivering 0,02 ml, 0,05 ml, 0,2 ml, 1,0 ml and 2,0 ml.

5.6 Graduated pipettes, capable of delivering 5 ml and 10 ml, graduated in 0,1 ml divisions.

5.7 Glass filter funnel, of diameter about 7 cm.

5.8 Filter paper, medium fast grade, of diameter about 15 cm, free from lactic acid and lactates.

5.9 Glass rod.

5.10 Plastic paddles, capable of mixing the sample-enzyme mixture in the spectrometric cell.

5.11 Spectrophotometer, capable of measuring at 340 nm, equipped with cells of optical path length 1 cm.

5.12 Parafilm$^{TM1)}$.

6 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707 | IDF 50.

Store the sample in such a way that deterioration and change in composition are prevented.

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1) Parafilm^{TM} is an example of a product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO or IDF of this product.

7 Preparation

7.1 Preparation of test sample

Transfer the test sample to a container with capacity about twice the volume of the sample and provided with an airtight lid. Close the container immediately. Mix the sample thoroughly by repeatedly shaking and inverting the container.

During preparation, avoid exposure of the test sample to the atmosphere in order to minimize adsorption of water.

7.2 Test portion

Weigh, to the nearest 1 mg, 1,0 g of the test sample in a 50 ml glass beaker (5.2).

7.3 Blank test

Carry out a blank test by proceeding as specified in 7.4 and 8.2, using all reagents but omitting the test portion.

7.4 Preparation of solution and deproteination

7.4.1 Dissolve the test portion (7.2) in about 20 ml of water preheated to between 40 °C and 50 °C, while stirring with the glass rod (5.9) or suitable means. Transfer the contents of the glass beaker quantitatively to a 100 ml one-mark volumetric flask (5.4) by rinsing the beaker with water. Cool the contents of the flask to about 20 °C.

7.4.2 Add to the solution (7.4.1), in the following order, 5,0 ml of potassium hexacyanoferrate(II) solution (4.1), 5,0 ml of zinc sulfate solution (4.2) and 10,0 ml of sodium hydroxide solution II (4.3.2), swirling thoroughly after each addition. Dilute with water to the 100 ml mark. Mix thoroughly and allow the mixture to stand at room temperature for 30 min.

7.4.3 Filter through a filter paper (5.8), discarding the first fraction of the filtrate.

Use of a centrifuge is a suitable alternative to filtration.

8 Procedure

CAUTION — Avoid contamination, especially with perspiration.

8.1 Test to check the activity of reagents

8.1.1 Whenever a new batch of reagents (4.6 to 4.10 inclusive) is prepared, or when such reagents have been kept in a refrigerator without being used for more than 2 weeks, or when restarting analytical work after a period of analytical inactivity, or whenever other conditions may justify it, perform the following test for the recovery of lactates.

8.1.2 Pipette 10 ml of lithium L-lactate solution (4.11) into each of two 100 ml one-mark volumetric flasks (5.4). Pipette 10 ml of lithium D-lactate solution (4.12) into each of two other 100 ml one-mark volumetric flasks (5.4). Determine the L-lactic acid and lactates content and the D-lactic acid and lactates content of the solutions in the two pairs of 100 ml flasks, proceeding as specified in 7.4.2, 7.4.3 and 8.2.

8.1.3 Calculate the lithium lactate content, w_L, expressed in milligrams per litre, using one of the following equations:

a) for the L-lactate solution:

$$w_L = 341 \times A$$

b) for the D-lactate solution:

$$w_L = 346 \times A$$

where

A is the numerical value of the absorbance at 340 nm, calculated in accordance with 8.2.1 and 8.2.2;

341 is the numerical value of the factor after substituting the molecular mass of lithium L-lactate (M_r = 96,1) and the final volume (V_1 = 2,24 ml) in 9.1 when L-lactate recoveries are evaluated;

346 is the numerical value of the factor after substituting the molecular mass of lithium D-lactate (M_r = 96,1) and the final volume (V_1 = 2,27 ml) in 9.1 when D-lactate recoveries are evaluated.

8.1.4 Taking into consideration the purity of the lithium L-lactate and lithium D-lactate used to prepare the solutions, the recovery of lithium L- or D-lactate from any of the flasks (8.1.2) shall be within the range 100 % ± 5 %. If the recoveries are not within this range, check the reagents, the operating technique, the accuracy of the pipettes and the condition of the spectrophotometer. Take the required action to obtain appropriate results. Repeat the test until satisfactory results are obtained.

8.2 Determination

8.2.1 Transfer using the required pipette (5.5) into the 1 cm cell of the spectrophotometer (5.11) according to the scheme in Table 1.

Table 1 — Procedure scheme

Pipette into spectrophotometer cells	Blank	D-Lactate standard	L-Lactate standard	Sample
Distilled water	1,000 ml	—	—	—
Standard (8.1.2)	—	1,000 ml	1,000 ml	—
Sample filtrate (7.4.3)	—	—	—	1,000 ml
Buffer solution, pH 10 (4.6)	1,000 ml	1,000 ml	1,000 ml	1,000 ml
NAD solution (4.7)	0,200 ml	0,200 ml	0,200 ml	0,200 ml
GPT suspension (4.10)	0,020 ml	0,020 ml	0,020 ml	0,020 ml
Mix the contents of the cell using a plastic paddle (5.10) or cover the 1 cm cell with parafilm (5.12) and invert several times. After mixing, leave the cell and its contents for 5 min before measuring the absorbance (A_{b0} and A_{s0}) against water at a wavelength of 340 nm.				
L-LDH suspension (4.8)	0,020 ml	—	0,020 ml	0,020 ml
D-LDH suspension (4.9)	0,050 ml	0,050 ml	—	0,050 ml
Exactly 45 min after mixing, measure the absorbance of the test solution (A_{b45} and A_{s45}) against water at a wavelength of 340 nm. Leave the cell again and after exactly 60 min after mixing measure the absorbance of the test solution (A_{b60} and A_{s60}) again against water at a wavelength of 340 nm.				

The L- or D-lactic acid and lactates content may be determined separately by adding either L-LDH (4.8) or D-LDH (4.9).

When only L-lactic acid and lactate are measured, the absorbance may be measured after 30 min and 45 min, respectively, after mixing.

8.2.2 Calculate the net absorbance value, A, to be used in the calculation (9.1) by using the following equation:

$$A = [(A_{s60} - A_{s0}) - 4(A_{s60} - A_{s45})] - [(A_{b60} - A_{b0}) - 4(A_{b60} - A_{b45})] \quad (1)$$

where

A_{s60} is the numerical value of the absorbance of the test solution measured after 60 min in 8.2.1;

A_{s0} is the numerical value of the absorbance of the test solution measured in 8.2.1;

A_{s45} is the numerical value of the absorbance of the test solution measured after 45 min in 8.2.1;

A_{b60} is the numerical value of the absorbance of the blank test solution measured after 60 min in 8.2.1;

A_{b0} is the numerical value of the absorbance of the blank test solution measured in 8.2.1;

A_{b45} is the numerical value of the absorbance of the blank test solution measured after 45 min in 8.2.1.

A slowly proceeding side reaction may occasionally occur. The contribution to the absorbance caused by this side reaction may be eliminated by extrapolation to the absorbance at time zero.

When only L-lactic acid and lactate are measured (see 8.2.1), the absorbance may be measured after 30 min and 45 min, respectively. In this case, the equation is changed as follows:

$$A = [(A_{s45} - A_{s0}) - 3(A_{s45} - A_{s30})] - [(A_{b45} - A_{b0}) - 3(A_{b45} - A_{b30})] \quad (2)$$

where

A_{s30} is the numerical value of the absorbance of the test solution measured after 30 min in 8.2.1;

A_{b30} is the numerical value of the absorbance of the blank test solution measured after 30 min in 8.2.1.

8.2.3 If the increase in absorbance calculated according to 8.2.2 exceeds 0,500 units, repeat the procedures specified in 8.2.1 through 8.2.3, using an appropriate aqueous dilution of the filtrate from both the test portion (7.4.3) and the blank test solution (7.3).

9 Calculation and expression of results

9.1 Calculation

Calculate the lactic acid and lactates content, w_L, expressed as milligrams of lactic acid per 100 g of non-fat solids, by using the following equation:

$$w_L = \left(\frac{A \cdot M_r}{k \cdot l \cdot m}\right) \times \left(\frac{V_1 \cdot V_4 \cdot V_5}{V_2 \cdot V_3}\right) \times \left(\frac{100}{w_s}\right) \times 10^5 \quad (3)$$

where

A is the numerical value of the absorbance at 340 nm, calculated in accordance with 8.2.2;

M_r is the relative molecular mass of lactic acid (M_r = 90,1);

k is the molar absorption coefficient of NADH at 340 nm, i.e. 6,3 × 10^6 cm^2/mol;

l is the optical path length of the spectrophotometric cells (l = 1 cm), in centimetres;

m is the mass of the test portion (7.3), in grams;

V_1 is the total volume of liquid in the spectrophotometric cell (see 8.2.1), in millilitres:

— when both L- and D-lactic acid and lactates are determined V_1 = 2,29 ml,

— when only L-lactic acid and lactates are determined V_1 = 2,24 ml,

— when only D-lactic acid and lactates are determined V_1 = 2,27 ml;

V_2 is the volume of the filtrate (see 7.4.3) in the spectrophotometric cell (see 8.2.1), in millilitres;

V_3 is the volume of the filtrate (see 7.4.3) taken for the dilution (see 8.2.3), if appropriate;

V_4 is the volume of the obtained solution in 7.4.2 (i.e. V_4 = 100 ml), in millilitres;

V_5 is the volume to which the test solution was diluted (see 8.2.3), if appropriate, in millilitres;

w_s is the non-fat solids content of the sample, expressed as mass fraction in percent.

NOTE The determination of fat is not part of the method specified in this International Standard. A recommended method for the determination of fat in dry milk is given in ISO 1736.

9.2 Expression of results

Express the test results in whole figures.

10 Precision

10.1 Interlaboratory test

The values for the repeatability and reproducibility were derived from the result of an interlaboratory test carried out in accordance with ISO 5725[2)].

Details of the interlaboratory test on the precision of the method have been published (see Reference [5]). The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

10.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases be greater than the following:

a) for an arithmetic mean of lactic acid and lactates content $\leqslant$ 60 mg per 100 g of non-fat solids: 10 mg/100 g;

b) for an arithmetic mean of lactic acid and lactates content $>$ 60 mg per 100 g of non-fat solids: 15 % (relative) of the arithmetic mean.

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2) ISO 5725:1986, *Precision of test methods — Determination of repeatability and reproducibility for a standard test method by inter-laboratory tests* (now withdrawn) was used to obtain the precision data.

10.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than the following:

a) for an arithmetic mean of lactic acid and lactates content $\leqslant$ 100 mg per 100 g of non-fat solids: 15 mg/100 g;

b) for an arithmetic mean of lactic acid and lactates content $>$ 100 mg per 100 g of non-fat solids: 20 % (relative) of the arithmetic mean.

11 Test report

The test report shall specify:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, with reference to this International Standard;

d) all operational details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

e) the test result(s) obtained and, if the repeatability has been checked, the final quoted result obtained.

Annex A
(normative)

Good laboratory practice (GLP) rules for the performance of enzymatic analyses

A.1 Introduction

Good Laboratory Practice rules for enzymatic analyses are less well known than those for other chemical analyses. Attention needs to be directed to such rules in order to obtain results with a satisfactory accuracy and precision. Prior to analyses, read the GLP rules described below.

A.2 Reagents

A.1.1 Use only enzymes of the prescribed grade (specific activity, concentration, contaminants with enzymatic activities, solvent).

A.1.2 Use only coenzymes of the prescribed grade (purity grade, salt or acid form, contaminants).

A.1.3 All reagents other than the enzymes and the coenzymes shall be of analytical grade.

A.1.4 The water for the preparation of the enzyme solutions and the other reagents shall be doubly glass-distilled.

A.1.5 The water for the preparation of the sample solutions shall be glass-distilled or deionized.

A.1.6 Store reagents and enzyme suspensions/solutions according to the instructions (usually between 2 °C and 8 °C).

A.1.7 Do not freeze enzyme suspensions.

A.1.8 When the expiry date of a reagent has been exceeded, either discard the reagent or check the efficiency of this reagent by examining standard solutions with varying amounts of analyte. The obtained absorbances shall be proportional to the concentrations.

A.1.9 Buffer solutions taken from the refrigerator shall be warmed up to room temperature before being added to the assay mixture.

A.3 Photometric and spectrophotometric cells

A.3.1 Use glass or plastic cells with an optical path length of 1 cm.

NOTE Plastic cells have the following advantages over glass cells:

a) they are cheap (disposable);

b) greater numbers of analyses are possible;

c) within one batch, plastic cells agree very well with regard to the absorbance.

A.3.2 Whenever a new batch of cells is put into use, check their optical path length against that of a precision cells (e.g. quartz cell), as follows.

Fill the precision cell and plastic cells with water and measure the absorbance (A_1) of each cell against water. Fill the cells, after rinsing, with a solution of NADH (approximately 0,15 mg/ml) and again measure the absorbance (A_2) against water. For both the precision cell and the plastic cells, calculate $A_2 - A_1$. If the difference ($A_2 - A_1$) between the two cell types exceeds 0,5 % of the net absorbance measurement for the precision cell, calculate the average percentage difference and take this into account for the path length, l, in Equation (3).

A.3.3 Always use clean and unscratched cells. Dry or clean the optical sides of the cells with a soft tissue only.

A.3.4 It is advisable not to measure the absorbance of the sample test cells against that of the blank test cell, since no information will be obtained about the order of magnitude of the absorbance of the blank test itself. Measure the absorbance of both the sample and the blank test cell against water and calculate the difference.

A.3.5 Do not measure the absorbance of a sample or blank test cell against an empty cell (because of light diffusion).

A.3.6 Mix the contents of the cell with a plastic paddle or by sealing the cell with parafilm and gently swirling.

A.3.7 Remove air bubbles from the walls of the cells using a paddle. Avoid scratching the optical side of the cell.

A.3.8 Always use the same kind of cells for the measurement of the sample test and blank test.

A.3.9 Always place glass or quartz cells in the same position in the cell holder. For this purpose, mark one optical side of the cell.

A.4 Photometers and spectrophotometers

A.4.1 Use a spectrophotometer (bandwidth $\leqslant$ 10 nm), a filter photometer provided with an interfering filter (bandwidth $\leqslant$ 10 nm), or a spectrum line photometer equipped with a mercury vapour lamp. Measurements carried out using a spectrophotometer or filter photometer shall be made at the absorption maximum of NADH or NADPH, i.e. 340 nm. Those carried out using a spectral line photometer with a mercury vapour lamp shall be made at 365 nm or 334 nm.

NOTE The molar absorption coefficients of NADH and NADPH measured at 334 nm, 340 nm and 365 nm are as follows:

a) NADH and NADPH at 334 nm (Hg): $6{,}18 \times 10^6$ cm^2/mol;

b) NADH and NADPH at 340 nm: $6{,}3 \times 10^6$ cm^2/mol;

c) NADPH at 365 nm (Hg): $3{,}5 \times 10^6$ cm^2/mol;

d) NADH at 365 nm: $3{,}4 \times 10^6$ cm^2/mol.

A.4.2 A linear relationship up to an absorbance of 2,0 shall exist between the absorbance and the concentration of NADH or NADPH. Check the linearity as follows:

a) pipette 2,00 ml of distilled water into a cell and measure the absorbance A_0 against water;

b) pipette 0,10 ml of NADH solution (0,5 mg/ml) into the cell; mix the contents of the cell and measure the absorbance A_1.

Calculate the reduced absorbance, A_{rn}, by using the following formula:

$$A_{r1} = \left(A_1 - A_0\right) \times \frac{2,1}{3,5}$$

Repeat the linearity check procedure 14 times, as described above.

After each pair of measurements, calculate the reduced absorbance, A_{rn}, by using the following formula:

$$A_{rn} = \left(A_n - A_0\right) \times \frac{V}{3,5}$$

where

A_n is the absorbance obtained at measurement n;

V is the volume of the cell contents at measurement n.

For each measurement, plot the volume of NADH solution present in the cell against the corresponding reduced absorbances. The correlation value of the measurements should be $>$ 0,99.

A.5 Automatic pipettes and other dispensers

A.5.1 Use automatic pipettes and other dispensers in accordance with the manufacturer's instructions.

A.5.2 Use the appropriate tips for each pipette.

A.5.3 Check the specifications of volume and repeatability of automatic pipettes and other dispensers periodically (e.g. monthly) as follows.

Weigh a glass beaker with water at time t. Pipette or dispense 1 $\times$ measure of water into the beaker and weigh exactly at (t + 1) min after the first weighing. Repeat the pipetting or dispensing procedure nine times. Weigh the beaker, without pipetting or dispensing, at the moment (t + 11), (t + 12), (t + 13), (t + 14) and (t + 15) min. Calculate from these weighings the evaporation loss per minute. Calculate the volume and repeatability of the pipette or dispenser, taking into account the loss of water by evaporation.

A.5.4 Transfer of heat from the palm of the hand during prolonged use can affect the volume of some automatic pipettes.

Check this phenomenon by the procedure described in A.5.3 and avoid the use of such pipettes.

A.5.5 Just before use, rinse the tip of the pipette several times with the solution/suspension to be delivered. For each sample solution, use a new pipette tip.

A.5.6 Pipette the sample, buffer, enzyme, coenzyme and sample solution, while inserting the tip as low as possible, into different corners of the cell.

Small amounts of enzyme solutions/suspension (10 – 50) µl may be pipetted onto the paddle, brought into the cell and mixed with the cell contents by the paddle.

A.5.7 Avoid contamination.

A.6 Other useful information

A.6.1 Check for possible interference and for gross errors by determining the absorbances of two solutions with different analyte concentrations. The absorbances obtained shall be proportional to the analyte concentration.

A.6.2 Use a standard to check the enzymatic reaction(s). This standard shall be considered as a working standard.

NOTE Reference materials having a certified purity can be obtained from organizations such as the National Institute of Standards and Technology (NIST) or the European Community Bureau of Reference (BCR).

A.6.3 Carry out a recovery test in the presence of the sample solution. The amount of analyte added shall be about the same as that already present in the sample solution.

A.6.4 Use one plastic paddle per cell or use each paddle once only.

NOTE The amount of liquid remaining on the paddle can be considered negligible.

Bibliography

[1] ISO 707 | IDF 50, *Milk and milk products — Guidance on sampling*

[2] ISO 1736, *Dried milk and dried milk products — Determination of fat content — Gravimetric method (Reference method)*

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[5] LEENHEER J. and JANS J.A. *Bulletin of the IDF*, No. 207, 1986, pp. 122-132

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